When Things Go Wrong

Contingency & Adjustment Protocol

A Companion Document to the 3R Senolytic Study Protocol

Anticipating Unexpected Results and Planning Adjustments

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Introduction: Why Plan for Failure?

In science, the most interesting results are often the ones you didn't expect. A good researcher doesn't just hope for the best — they prepare for every possible outcome. This document is a "what-if" guide: for each measurement in the 3R study, we imagine the worst-case scenario and plan exactly what to do about it.

Think of this as a GPS that recalculates the route when you miss a turn. If the biomarkers don't move in the expected direction, we don't panic — we adjust.

How to Use This Document

Each section follows the same structure:

- **The Expected Result**: what we think will happen
- **The Scenario**: what actually happened (the surprise)
- **Why It Might Happen**: the biological reasoning
- **The Adjustment**: what we change in the protocol

Scenario 1: p16 and p21 Don't Decrease

Expected Result

$p16^{INK4A}$ and p21 mRNA levels in blood should decrease in the treatment group as fisetin kills senescent cells.

> ⚠️ **What If This Happens?**
>
> **The Problem**: After 16 weeks, p16 and p21 levels are the same in both groups. Or worse — they went UP slightly in the treatment group.

Possible Reasons

1. **Fisetin dose is too low**: 500mg may not be enough to trigger apoptosis of senescent cells in humans. Mouse doses are typically ~100mg/kg, which would be ~7,000mg for a 70kg human. We chose 500mg based on conservative safety data, but it may be sub-therapeutic.
2. **Blood p16 doesn't reflect tissue p16**: We're measuring p16 mRNA in circulating blood cells (mostly leukocytes). These are immune cells, not the fibroblasts or endothelial cells we actually want to target. Immune cells may have different senescence dynamics.
3. **The 2-day pulse is too short**: Maybe senescent cells need 3-4 consecutive days of fisetin exposure to undergo apoptosis.
4. **Fisetin is poorly absorbed**: Even with piperine, oral fisetin has low bioavailability (~5-10%). Most of it may never reach the bloodstream.
5. **Transient increase after killing cells**: When senescent cells die, they release their contents — including p16 protein and mRNA — which could temporarily spike blood levels before clearing.

> 🔧 **Proposed Adjustment**
>
> ### Adjustments to Try
>
> **If p16 is unchanged:**
>
> - **Increase fisetin dose**: Try 1,000mg or 1,500mg per pulse day. Monitor liver enzymes closely (ALT/AST) at every visit.
> - **Extend pulse to 3 days**: Give fisetin for 3 consecutive days instead of 2. This increases total senolytic exposure by 50%.

- **Switch to liposomal fisetin**: Liposomal encapsulation can increase bioavailability 5-10×. If available, use liposomal fisetin at the same 500mg dose — it should deliver much more to tissues.
- **Add a tissue biomarker**: If blood p16 is unreliable, add an optional 3mm skin punch biopsy at baseline and Day 120. Measure p16 directly in skin fibroblasts where the action is.
- **Shorten the pulse interval**: Instead of every 15 days, try every 10 days (2 days on, 8 days off). More frequent killing cycles.

If p16 actually increased:

- This may be a transient spike from dying cells releasing their contents. Check the *timing* of the blood draw — if it's within 3-5 days of the last pulse, you may be catching the debris. Draw blood at least 7 days after the last pulse.
- If p16 remains elevated at Day 120 (>7 days after last pulse), consider that fisetin may be paradoxically inducing senescence in some cell populations at this dose. Reduce dose in follow-up study.

Scenario 2: IL-6 and IL-8 Increase (More Inflammation)

Expected Result

SASP markers IL-6 and IL-8 should decrease as senescent cells are eliminated and their toxic secretion stops.

⚠️ **What If This Happens?**

The Problem: IL-6 and IL-8 went UP in the treatment group. The placebo group stayed stable.

Possible Reasons

1. **The "dying zombie" effect**: When fisetin kills senescent cells, they rupture and dump their SASP contents (including IL-6 and IL-8) into the surrounding tissue and bloodstream. This is a *transient* spike — like demolishing a building releases dust. The inflammation should resolve within days.
2. **NMN activating immune cells**: NAD+ can activate certain immune cells (especially macrophages) which produce IL-6. More NAD+ = more active immune system = more inflammation markers.
3. **Spermidine at wrong dose**: Too much spermidine can overstimulate the immune system.
4. **Real tissue damage**: The combination could be causing low-grade liver or gut irritation, triggering systemic inflammation.

🔧 **Proposed Adjustment**

Adjustments to Try

If IL-6/IL-8 spike at Day 30 but normalize by Day 60:

- This is the expected "clearance phase." The first few pulses kill the most senescent cells, releasing the most SASP. As the senescent cell burden decreases, subsequent pulses release less. **No adjustment needed** — but add a time-course sub-study: draw blood at Day 3 (during first pulse), Day 5, Day 7, Day 10 to map the spike and recovery.

If IL-6/IL-8 remain elevated through Day 120:

- **Check liver enzymes**: If ALT/AST are elevated, the supplements may be causing liver stress. Reduce fisetin dose or halt the study.

- **Remove NMN temporarily**: Test whether NMN is the inflammatory trigger. If removing NMN normalizes IL-6, switch to NR (Nicotinamide Riboside) which may be less inflammatory.
- **Add an anti-inflammatory bridge**: Low-dose curcumin (500mg) or omega-3 EPA/DHA (2g) on non-pulse days to counteract the inflammatory spike without interfering with the senolytic mechanism.
- **Measure CRP and TNF-α**: Add these broader inflammatory markers to distinguish "senescent cell clearance" inflammation from "tissue damage" inflammation.

Scenario 3: NAD+ Levels Don't Increase

Expected Result

Blood NAD+ should increase in the treatment group within 2-4 weeks of daily NMN supplementation (250mg/day).

> ⚠️ **What If This Happens?**
>
> **The Problem**: NAD+ levels are unchanged or barely increased after 16 weeks of NMN.

Possible Reasons

1. **NMN is being degraded before absorption**: NMN is unstable in the acidic stomach environment. If taken without food or in non-enteric capsules, much of it may be destroyed before reaching the intestine.
2. **CD38 is eating all the NAD+**: CD38 is an enzyme that degrades NAD+. Its expression increases with age. If participants have high CD38 activity, the NAD+ produced from NMN is immediately destroyed.
3. **250mg is too low**: Some human studies use 300-500mg NMN. Our conservative dose may be sub-threshold for older adults with already-depleted NAD+.
4. **Wrong NAD+ measurement**: We're measuring total blood NAD+, but most NAD+ is inside cells (intracellular). Plasma NAD+ may not reflect intracellular levels.
5. **Poor quality NMN**: Not all NMN supplements are equal. Some contain mostly nicotinamide (cheaper, less effective) rather than true NMN.

> 🔧 **Proposed Adjustment**
>
> ### Adjustments to Try
>
> - **Increase NMN dose to 500mg/day**: This is still well within the safe range used in human studies.
> - **Switch to sublingual NMN**: Sublingual (under the tongue) absorption bypasses the digestive system entirely, delivering NMN directly into the bloodstream.
> - **Switch to NR (Nicotinamide Riboside)**: NR is more stable than NMN and has better oral bioavailability. Try 300mg NR instead of 250mg NMN.

- **Add apigenin or quercetin**: These are natural CD38 inhibitors. By blocking CD38, they prevent NAD+ degradation, allowing the NMN-derived NAD+ to actually accumulate. Dose: apigenin 50mg or quercetin 500mg daily.
- **Verify NMN purity**: Send a sample to a third-party lab (e.g., Labdoor, Eurofins) to verify it's genuine NMN, not nicotinamide. Require a Certificate of Analysis (CoA) from the supplier.
- **Measure intracellular NAD+**: Switch from plasma to whole-blood NAD+ assay (which includes intracellular content from lysed red blood cells).

Scenario 4: Autophagy Markers Don't Change

Expected Result

LC3-II/I ratio should increase and p62 should decrease, indicating active autophagy from spermidine supplementation.

> ⚠️ **What If This Happens?**
>
> **The Problem**: LC3-II/I and p62 are unchanged in both groups.

Possible Reasons

1. **3mg spermidine is too low**: Dietary spermidine intake varies from 5-20mg/day depending on diet. Adding 3mg may be lost in the noise of normal dietary variation.
2. **Autophagy is transient**: Spermidine induces a brief burst of autophagy (hours), not a continuous state. If blood is drawn days after the last dose, the autophagy signal is gone.
3. **LC3-II and p62 in blood cells don't reflect tissue autophagy**: Same problem as blood p16 — we're measuring the wrong tissue.
4. **mTOR is too active**: If participants eat high-protein meals (which activate mTOR and suppress autophagy), the spermidine signal may be overridden.

> 🔧 **Proposed Adjustment**
>
> ### Adjustments to Try
>
> - **Increase spermidine to 6mg or 9mg/day**: Still very safe (dietary intake can be 20mg+).
> - **Measure autophagy at the right time**: Draw blood 4-6 hours after the morning dose, when spermidine-induced autophagy should peak.
> - **Add an autophagy flux assay**: Instead of static LC3/p62, measure autophagic flux (the rate of autophagy) by comparing with and without a lysosomal inhibitor (bafilomycin). This requires cell culture from blood samples.
> - **Ask participants to fast before blood draws**: 12-hour overnight fast eliminates the mTOR-activating effect of food, giving spermidine a clean slate to induce autophagy.

- **Combine with intermittent fasting protocol**: Ask participants in the treatment group to follow 14:10 intermittent fasting (14h overnight fast, 10h eating window). Fasting + spermidine should synergize for autophagy induction.

Scenario 5: SDF1 Doesn't Increase

Expected Result

SDF1 should increase as NMN restores sirtuin activity, which we hypothesized would normalize SDF1 expression.

⚠️ **What If This Happens?**

The Problem: SDF1 is unchanged after 16 weeks.

Possible Reasons

1. **The sirtuin→SDF1 link doesn't exist or is indirect**: We hypothesized that sirtuins regulate SDF1, but this was inference, not proven fact. SDF1 regulation may be controlled by other pathways (NF-κB, HIF-1α).
2. **SDF1 in blood doesn't reflect skin SDF1**: Yoon measured SDF1 in tissue biopsies, not blood. Blood SDF1 may be dominated by bone marrow and liver production, not skin.
3. **SDF1 takes longer to recover**: Maybe the senescent fibroblasts that produce low SDF1 must be killed AND replaced by new fibroblasts before SDF1 normalizes. Cell turnover in skin takes 28+ days, but deep dermal fibroblasts turn over much slower (months to years).

🔧 **Proposed Adjustment**

Adjustments to Try

- **Measure SDF1 in skin biopsy**: If participants consent to a 3mm punch biopsy, measure SDF1 protein directly in the tissue where it matters. Blood SDF1 may be a poor proxy.
- **Extend the study to 6-9 months**: If SDF1 recovery requires new fibroblast generation, 4 months may not be enough. Plan a 6-month extension study for participants who show other positive signals.
- **Measure NF-κB activity**: If SDF1 is regulated by NF-κB (not sirtuins), we need to track NF-κB. Add a NF-κB p65 ELISA or reporter assay.
- **Accept that SDF1 may not be the right blood biomarker**: Drop it from primary endpoints and focus on p16 and IL-6 instead. Not every hypothesis can be validated — that's science.

Scenario 6: Skin Spots Don't Improve

Expected Result

Brown spots should decrease in size and darkness in the treatment group, based on the Yoon (2018) mechanism.

> ⚠️ **What If This Happens?**
>
> **The Problem**: Photographs show no visible difference in brown spots between treatment and placebo groups.

Possible Reasons

1. **16 weeks is too short**: Skin pigmentation changes slowly. Melanin in the epidermis takes ~28 days to shed, but the deep dermal melanin (where the senescent fibroblasts live) may take 6-12 months to clear.
2. **The problem is deeper than we can reach**: Solar lentigines have pigmentation in BOTH the epidermis and dermis. Topical creams and even systemic treatments may clear the epidermal pigment but not the deep dermal pigment.
3. **Photography isn't sensitive enough**: Visible changes of 5-10% may not be detectable with standard photography, even with controlled lighting.
4. **Sun exposure during the study**: If participants don't use sunscreen daily, new UV damage masks the treatment effect.
5. **Fisetin doesn't reach skin fibroblasts**: Oral fisetin may preferentially accumulate in liver and kidney, not skin. Skin blood flow is relatively low compared to these organs.

> 🔧 **Proposed Adjustment**
>
> ### Adjustments to Try
>
> - **Extend to 6-9 months**: Skin changes from within take time. A 9-month study would capture ~9 skin renewal cycles.
> - **Use cross-polarized photography**: This technique sees through the shiny surface layer and reveals deeper pigmentation changes invisible to standard photography.
> - **Mandatory daily sunscreen**: Require all participants (both groups) to use SPF 50+ sunscreen daily. This prevents new UV damage from confounding the results.

- **Add topical fisetin**: Apply a fisetin-containing cream directly to one brown spot (leaving another untreated as internal control). This bypasses the bioavailability problem by delivering fisetin directly to the target tissue.
- **Add a skin biopsy for melanin quantification**: Quantify melanin content biochemically rather than relying on photo analysis. Masson-Fontana staining gives precise melanin measurements.
- **Consider that skin improvement may require combination with dermatological procedures**: The 3R approach may need to be combined with microneedling (as in Yoon's study) for visible results. The supplements clear senescent cells systemically; the microneedling provides the physical stimulus for tissue remodeling.

Scenario 7: No Difference Between Groups At All

Expected Result

The treatment group should outperform placebo on at least some biomarkers.

> ⚠️ **What If This Happens?**
>
> **The Problem**: Every single measurement shows no statistically significant difference between treatment and placebo. Both groups improved equally (or didn't change at all).

Possible Reasons

1. **Strong placebo effect**: When people know they're in a health study, they unconsciously change behaviors — eating better, sleeping more, exercising more. This "study effect" can mask small treatment differences.
2. **Doses are all sub-therapeutic**: We chose very conservative doses for safety. If none are high enough to produce a biological effect, we'd see no difference.
3. **The combination cancels itself out**: Perhaps fisetin and NMN have opposing effects on some pathway. For example, fisetin is a mild SIRT1 inhibitor at high doses, while NMN activates SIRT1. They could neutralize each other.
4. **40 people is too few**: If the real effect size is small (Cohen's d ~0.3), we'd need ~175 people per group to detect it. Our pilot is powered only for large effects.
5. **The supplements were not taken correctly**: Participants may have missed doses, taken them at wrong times, or stored them improperly (NMN degrades in heat and humidity).

> 🔧 **Proposed Adjustment**
>
> ### Adjustments to Try
>
> - **Check adherence first**: Count returned capsules at each visit. If adherence is <80%, the study didn't test the treatment — it tested non-adherence. Use electronic pill bottles (with timestamped opening) in the next study.
> - **Measure supplement blood levels**: Add a pharmacokinetic sub-study on a subset (n=5) — measure fisetin, NMN metabolites, and spermidine in blood 2 hours after dosing. If they're not in the blood, the supplements aren't being absorbed.

- **Increase ALL doses**: If safety allows, increase to fisetin 1,000mg, NMN 500mg, spermidine 6mg in the follow-up study.
- **Test components individually**: Run a 3-arm follow-up study: (A) fisetin alone, (B) NMN alone, (C) combination. This reveals if one component is negating another.
- **Enroll older/frailer participants**: 50-55 year olds may have too few senescent cells to show a measurable difference. Try 65-80 year olds with documented age-related conditions.
- **Accept the null hypothesis gracefully**: If a well-powered study shows nothing, that's still valuable science. Publish it. The community learns as much from negative results as from positive ones.

Scenario 8: Liver Enzymes Elevate

Expected Result

Liver function tests (ALT, AST) should remain within normal range throughout the study.

⚠️ **What If This Happens?**

The Problem: One or more participants show elevated ALT/AST (>2× upper limit of normal) at Day 30 or later.

Possible Reasons

1. **Fisetin hepatotoxicity**: At high doses, some flavonoids can stress the liver. Fisetin has not shown this in animal studies, but human responses differ.
2. **NMN liver stress**: High-dose niacin (a NAD+ precursor) is known to elevate liver enzymes. NMN may have a similar effect in susceptible individuals.
3. **Unrelated cause**: The participant may have started a new medication, consumed alcohol, or caught a viral hepatitis.
4. **Pre-existing condition**: The participant may have had mild fatty liver disease that wasn't caught in screening.

🔧 **Proposed Adjustment**

Adjustments to Try

Immediate action if ALT/AST >3× normal:

- Stop all study supplements for that participant immediately
- Repeat liver panel within 7 days
- If normalizing → likely supplement-related, document as adverse event, do not restart
- If not normalizing → refer to hepatologist, investigate other causes

For the study protocol:

- Add liver panel (ALT, AST, bilirubin, GGT) to EVERY study visit, not just baseline and end
- Set a pre-specified stopping rule: if >2 participants develop ALT >3× ULN, pause and review
- In follow-up study: split the dose (half AM, half PM) to reduce peak liver exposure

- Consider enteric-coated capsules to bypass the liver's first-pass metabolism

Scenario 9: Partial Response — Some Biomarkers Move, Others Don't

⚠️ **What If This Happens?**

The Problem: IL-6 decreases nicely (good!), NAD+ increases (good!), but p16 is unchanged and skin spots don't improve.

What This Means

This is actually the **most likely outcome** and is scientifically very informative. It tells us:

- The supplements ARE doing something biological (IL-6 and NAD+ prove bioactivity)
- The senolytic effect (fisetin killing zombie cells) may be weaker than the metabolic effect (NMN boosting NAD+)
- The study may need to be longer for downstream effects (skin changes follow biomarker changes by months)

🔧 **Proposed Adjustment**

Adjustments to Try

Treat the 3R components as independent levers:

- If NAD+ increases but p16 doesn't change → NMN works (REPAIR) but fisetin doesn't (REMOVE fails). Increase fisetin, keep NMN.
- If IL-6 decreases but NAD+ doesn't → spermidine may be reducing inflammation (via autophagy) independent of senescence. That's still valuable — adjust the study narrative.
- If blood biomarkers improve but clinical endpoints don't → extend the study duration. Biology precedes visible change by months.

Create a "responder vs. non-responder" analysis:

- Some people may respond dramatically while others don't respond at all. Look at individual data, not just group averages.
- Check if responders share characteristics: younger? specific diet? specific gut microbiome? genetic variants?
- This personalized data is often more valuable than group means for designing the next study.

Scenario 10: The Placebo Group Improves More Than Expected

⚠️ **What If This Happens?**

The Problem: Both groups show similar improvements. The placebo group's p16 decreased by 15% and IL-6 by 10%. The treatment group's p16 decreased by 18% and IL-6 by 12%. The difference is not statistically significant.

What This Means

- **The "healthy volunteer effect"**: People who volunteer for health studies tend to be health-conscious. Being in a study makes them even more health-conscious. They eat better, sleep better, exercise more — and these lifestyle changes reduce senescent cell burden naturally.
- **The supplement information effect**: Even placebo participants may research "senescence" and "NMN" online, then start eating strawberries (natural fisetin source), taking other supplements, or fasting — confounding the results.
- **Regression to the mean**: People with high baseline p16 or IL-6 are more likely to naturally decrease toward their personal average at the next measurement.

🔧 **Proposed Adjustment**

Adjustments to Try

- **Use a run-in period**: Before randomization, have all participants take placebo for 2 weeks. This washes out the "study excitement" effect and identifies non-adherent participants before the real study starts.
- **Track lifestyle changes**: Add a weekly lifestyle questionnaire (diet, exercise, sleep, new supplements, medication changes). Statistically adjust for these confounders.
- **Use a crossover design** for the follow-up: After 16 weeks, take a 4-week washout, then switch groups (treatment becomes placebo, placebo becomes treatment). Each person serves as their own control, eliminating individual variation.
- **Stratify randomization by baseline senescence burden**: Ensure that participants with high p16 are equally distributed between groups. If most high-p16 people end up in placebo by chance, the treatment looks less effective.

Quick Reference: Troubleshooting Matrix

Problem	Most Likely Cause	First Adjustment	Escalation
p16 unchanged	Fisetin dose too low	Increase to 1,000mg	Switch to liposomal fisetin
IL-6 increased	Transient cell clearance spike	Draw blood 7+ days post-pulse	Add anti-inflammatory bridge
NAD+ unchanged	Poor absorption or CD38 degradation	Increase to 500mg, sublingual	Switch to NR, add apigenin
Autophagy unchanged	Spermidine dose too low / timing wrong	Increase to 6-9mg, fasted blood draw	Add autophagy flux assay
SDF1 unchanged	Blood ≠ tissue SDF1	Measure in skin biopsy	Drop as primary endpoint
Skin spots unchanged	Too short / too deep	Extend to 6-9 months, add sunscreen mandate	Add topical fisetin + microneedling
No group difference	Doses sub-therapeutic / sample too small	Check adherence + blood levels	3-arm individual component study
Liver enzymes elevated	Supplement hepatotoxicity	Stop supplements for that participant	Add liver panel to every visit
Partial response	Components have different potencies	Analyze responders vs. non-responders	Test components individually
Placebo improves equally	Healthy volunteer effect	Add run-in period + lifestyle tracking	Crossover design in follow-up

Decision Tree: What to Do After Day 30 Results

The Day 30 visit is your first checkpoint. Here's a decision tree based on what you see:

Proposed Adjustment

```
Day 30 Blood Draw Results
│
├── NAD+ increased?
│   ├── YES → NMN working. Continue.
│   └── NO → Increase NMN to 500mg. Check absorption. Switch to sublingual.
│
├── p16 decreased?
│   ├── YES → Fisetin working. Continue current protocol.
│   ├── NO → Check adherence (capsule count).
│   │   ├── Adherence OK → Increase fisetin to 1,000mg for next pulse.
│   │   └── Adherence poor → Counsel participant. Continue same dose.
│   └── INCREASED → Check timing of blood draw (must be 7+ days post-pulse).
│       If still elevated → Reduce fisetin dose, monitor closely.
│
├── IL-6 changed?
│   ├── DECREASED → SASP clearance working. Continue.
│   ├── UNCHANGED → Normal. May need more pulses. Continue.
│   └── INCREASED → Check liver enzymes.
│       ├── Liver OK → Transient clearance spike. Continue. Recheck at Day 60.
│       └── Liver elevated → STOP supplements for this participant. Investigate.
│
└── Overall assessment:
    ├── All biomarkers trending right → Continue full protocol.
    ├── Mixed signals → Continue, add skin biopsy at Day 60 for more data.
    └── All flat or wrong direction → Convene safety monitor. Consider dose escalation or early terminat
```

Closing Thoughts

The worst thing a researcher can do after an unexpected result is panic and change everything at once. The best thing is to change **one variable at a time**. If you increase fisetin AND switch to liposomal AND add apigenin all at once, and the result improves, you won't know which change caused it.

This is why pilot studies exist — not to prove something works, but to learn **how to test it properly** in the next, bigger study. Every unexpected result is a clue that makes the follow-up study better.

> 🔧 **Proposed Adjustment**
>
> **Golden Rules for Protocol Adjustment:**
>
> 1. Change ONE thing at a time
> 2. Document every change and why you made it
> 3. Pre-register adjustment decisions in the trial registry
> 4. If in doubt, choose safety over data
> 5. Talk to your participants — they notice things blood tests can't measure
> 6. Negative results are still results. Publish them.

"Science is not about being right. It's about finding out what's true."

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